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HYPOXIA LEVEL AND MATRIX METALLOPROTEINASES-2 AND -9 ACTIVITY IN LEWIS LUNG CARCINOMA: CORRELATION WITH METASTASIS

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Aim: **To investigate the relationship between tumor hypoxia *in vivo*, activity of matrix metalloproteinases (MMPs), and metastatic potential of tumor.** ***Materials and Methods:*** **Lewis lung carcinoma (3LL) was used in this study. Total activity of MMP-2 and -9 in tumor was measured biochemically, tumor hypoxia level was assessed by ^{31}P NMR spectroscopy in tissue perchloric extracts.** ***Results:*** **It was determined that hypoxia level in primary tumor has been concomitantly increasing along with tumor growth and correlated with metastasis level in lung. The positive correlation between hypoxia level and activities of MMP-2 and MMP-9 in primary tumor was registered. Moreover, the activity of MMP-2 and -9 in 3LL (primary tumor) directly correlates with metastasis level in lung.** ***Conclusion:*** **This study demonstrated that the growth of primary tumor is distinctly accompanied by an increase of tumor hypoxia level which positively correlates both with the activity of MMP-2 and -9 in primary tumor and metastatic efficiency.**
Key Words: **hypoxia, metastasis, matrix metalloproteinases, Lewis lung carcinoma, ^{31}P NMR spectroscopy.**

It is known that the one of the most characteristic features of malignant tissue is the high level of intratumoral hypoxia, and this phenomen is the most important in determining the differences between tumor and normal tissues as well as benign tumors [1, 2]. The experimental results permit to consider tumor hypoxia as a powerful factor for induction of angiogenesis [3] and to associate it with tumor progression and metastatic spread [4, 5]. Simultaneously with this it is necessary to pay attention to experimental findings that matrix metalloproteinases (MMPs) have close connections with metastatic phenotype of tumor cells *in vitro* and *in vivo* [6, 7]. There are rather clear evidences that there is the direct correlation between elevated level of tumor MMPs activity and tumor ability to metastasize [6, 8].

At the same time researchers do not pay due attention to the necessity to find out the character of relations between the level of hypoxia and MMP activities although there is an information concerning the enhancement of MMP-2 expression in the cells of liver upon hypoxia state *in vitro* [9]. It was also shown that hypoxia caused the increased MMP-1 and MMP-3 expression in rheumatoid synovial fibroblasts [10]. It was observed that human MDA-MB-231 breast carcinoma cells displayed increased MMP-9 secretion when cultured under reduced oxygen conditions [11]. Himelstein and Koch [12] have shown that hypoxia upregulates MMP-9 in alveolar rhabdomyosarcoma cell line. At the same time authors were unable to demonstrate a consistent hypoxia-mediated increase in MMP-9 protein, RNA, or transcriptional activity measured with special constructs and concluded that MMP-9 expression is not directly affected by exposure to hypoxia *in vitro*. Guo et al. [13] have shown that hypoxia did not affect the MMP-9 expression in esophageal cancer cell lines.

At the same time the determination of interactions between MMPs activities, the hypoxia levels and expression of HIF-1α in tumor is the most urgent scientific problem resolving of which will give the possibility to clarify the role of hypoxia-regulated proteins in metastasis and work out the methods of diagnosis and prognosis of disease outcome.

MATERIALS AND METHODS

Animals and tumor. Female C57BL/6 mice (IEPOR, NAS of Ukraine) weighting 22–25 g were used. Animals were kept in Makrolon cages bedded with dust-free wood granules and had free access to a standard diet and tap water. Transplanted Lewis lung carcinoma (3LL) was used in this study. The single-cell suspension of 3LL was injected intramuscularly into the leg (0.5×10^6 cells/animal). All experiments had been approved by the regional animal ethics committee.

^{31}P NMR spectroscopy. ^{31}P NMR spectra (121,5 MHz) of perchloric acid (PCA) tissue extracts (3LL and muscle of tumor-bearing mice) were recorded with a Mercury-300 BB Spectrometer (Varian, USA) equipped by Sparcs station 4, using a probe tube of 5 mm inner diameter. Perchloric acid (PCA) tissue extracts were prepared as described previously [14]. As a standard substance, methylenediphosphonic acid trisodium salt (Sigma, USA) was applied. The Pi/PCr, Pi/β NTP and PME/βNTP ratios were used as the most reliable and frequently applied ^{31}P NMR parameters for estimation of the changes in tissue hypoxia levels [15]. ^{31}P NMR spectra of muscle of tumor-bearing mice representing the "classic" ^{31}P NMR spectra were used as a control for

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Abbreviations used: 3LL – Lewis lung carcinoma; MMPs – matrix metalloproteinases; PCA – perchloric acid.

the procedure of PCA extraction for each tumor tissue sample.

Activity of MMPs. Total activity of matrix MMPs (type IV collagenases: MMP-2 (gelatinase A) and MMP-9 (gelatinase B)) was determined for each sample by substrate hydrolysis using the substrate gelatin (Sigma, USA). The activity assay was performed according to the assay protocol [16] with minor modifications. The concentration of protein residuals formed as a result of substrate proteolysis was determined by the method of Moore and Stein [17] with minor modifications. Results were expressed as relative units, i.e. the rate of gelatin hydrolysis.

Statistical analysis. Values given in this study are means ± SEM. Differences between the experimental points were evaluated by Student's *t*-test and considered significant for $p < 0.05$. Correlation assays have been performed using both Pearson's coefficient (r) and Spearman's coefficient (*rho*). Correlations were considered to be significant at p values of 0.05 or less.

RESULTS

Hypoxia level and growth of primary tumor. In the present study, we have notice that the changes in the ^{31}P NMR spectra correlate well with an increase in tumor volume: Pi/PCr, Pi/β NTP and PME/βNTP ratios directly correlated with the weight of tumor ($r = 0.84$, $p = 0.0088$; $r = 0.86$, $p = 0.001$; $r = 0.78$, $p = 0.017$, respectively): one may conclude that in parallel with tumor growth the hypoxic fraction of tumor became larger (Fig. 1).

Hypoxia level and activity of gelatinases in primary tumor. Our investigation provided also the possibility to establish the positive correlation between tumor hypoxia (Pi/PCr, Pi/βNTP and PME/βNTP ratios) and total MMP-2 and MMP-9 activities in tumor tissue: $r = 0.83$, $p = 0.01$; $r = 0.81$, $p = 0.014$; $r = 0.73$, $p = 0.035$. Fig. 2 clearly demonstrates that the increase of Pi/βNTP ratio in primary tumor, representing the enhancement of tumor hypoxia level, is accompanied by the increase of activity of gelatinases ($r_k = +0.81$, $p = 0.014$).

Activity of gelatinases in primary tumor and metastasis rate. In experiments *in vivo* with mice bearing 3LL it was shown that the extension of tumor growth is accompanied by an essential increase of

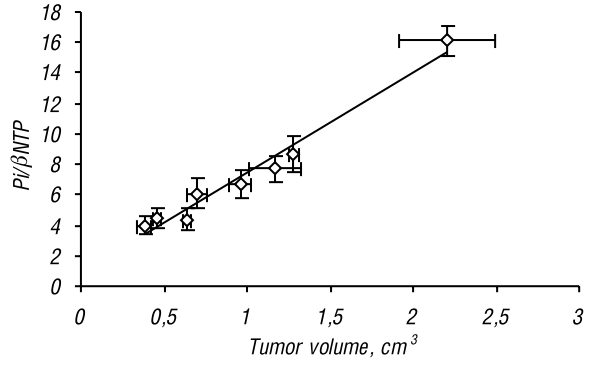

Fig. 1. Correlation between tumor volume and Pi/βNTP in primary 3LL. OX: tumor volume, cm^3; OY: Pi/βNTP ratio; values are mean ± SEM. $r = 0.86$, $p < 0.01$

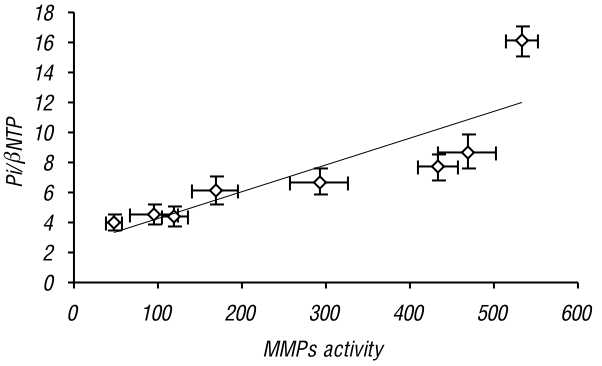

Fig. 2. Correlation between activity of gelatinases and Pi/βNTP in primary 3LL. OX: gelatinase activity, rel. U; OY: Pi/βNTP ratio; values are mean ± SEM. $r = 0.81$, $p < 0.01$

MMP-2 and MMP-9 activities in the primary tumor (Table 1). Analysis of the obtained data allowed to reveal and separate two groups of primary tumors according to MMPs activity and demonstrated a clear correlation between activities of MMPs in primary tumor and volume of metastasis (Table 2). These data obtained in experiments with metastatic tumors have shown the real association between MMPs activities in primary tumor and its metastatic potential.

Taking into account that tumors with larger volumes have the bigger hypoxic fraction and reveal higher activities of MMPs, that promote the metastasis spread, one may consider that obtained results confirm the known hypothesis that tumor hypoxia is the one of the main factors which initiate metastasis.

Table 1. Total activity of gelatinases in 3LL and parameters both of primary tumor growth and lung metastases

Day after tumor inoculation	Gelatinases activity (rel. U)	Volume of primary tumor (cm^3)	Number of lung metastases
12	47.0 ± 9	0.4 ± 0.1	2.3 ± 0.3
14	117.8 ± 12	0.8 ± 0.2	2.7 ± 0.5
16	166.7 ± 28	0.9 ± 0.2	2.7 ± 0.8
18	292.0 ± 35	1.1 ± 0.3	5.4 ± 1.0
20	434.2 ± 24	1.2 ± 0.2	11.6 ± 3.4
21	468.0 ± 35	1.4 ± 0.3	18.3 ± 7.8
24	534.4 ± 19	1.9 ± 0.5	16.8 ± 7.3
27	567.8 ± 48	2.3 ± 0.4	22.5 ± 12.0

$r_{2\text{-}3} = 0.918$, $p < 0.001$; $r_{2\text{-}4} = 0.92$, $p < 0.01$.

Table 2. Correlation between activity of gelatinases in primary tumor and indices of 3LL progression

Ranges of gelatinases activity (rel. U)	Volume of primary tumor (cm^3)	Number of metastases
0-300	0.7 ± 0.24	3.1 ± 1.1
300-650	1.7 ± 0.48	17.3 ± 7.6
	p < 0.05	p < 0.05

Correlation between the parameters were evaluated by Spearman-rho coefficient.

DISCUSSION

^{31}P NMR spectroscopy has been used for assessing tumor oxygen status indirectly from changes in ^{31}P NMR spectra, to estimate tumor hypoxia as a function of time after transplantation. Previous studies have clearly indicated a relationship between tumor oxygenation and metabolic status. Okunieff et al. [18] noted that an increase in the hypoxic fraction occurs in parallel with a decrease in high-energy phosphates. Significant correlations between hypoxic fraction and metabolic ratios measured by ^{31}P NMR spectroscopy were noted by Fu et al. [19] studying the EMT-6 murine tumor at different volumes. Vaupel et al.

[20] observed a significant correlation between the partial pressure of oxygen as measured using oxygen electrodes, and NMR-measured metabolic ratios and concluded that NMRS could be used to detect changes in tumor energetic induced by changes in tumor oxygenation.

It should be noted that we have evaluated the hypoxia level in tumor tissue by NMR spectroscopy. This technique was proposed and exploited to evaluate both tumor bioenergetic status and tumor oxygenation that are closely connected [15,18-20]. In spite of this, the method is considered as indirect tool for assessment of hypoxia extent, the advantages of this technique is the possibility to evaluate simultaneously the bioenergetics status and some other metabolic processes in tumor tissue. Meanwhile other methods are used for evaluation of hypoxia level although they cannot also be qualified as the direct methods. Nevertheless, they are considered as rather accurate for such task. It was reported about such intrinsic markers of hypoxia as pimonidasole [21], Glut-1 (glucose transporter) [22] and carbonic anhydrase (CA IX) [23]. Rofstad et al. [21] demonstrating the connection between metastasis and hypoxia level in primary tumor, have evaluated the hypoxia level by means of pimonidazole. It has been clearly shown that the assessment of Glut-1 expression in tumor may be justified in the identification of changes in gene expression associated with chronic tumor hypoxia and metastatic spread, despite the fact that the correlation between Glut-1 and tumor pO_2 measurement was weak [22]. Results of Loncaster et al. [23] suggest that CA IX expression reflects the enhanced metastatic potential of hypoxic tumors. Altogether, these studies distinctly demonstrate the direct association between the incidence of metastases and extent of hypoxia in the primary tumor.

There are many studies including clinical ones showing that tumor hypoxia may enhance the malignant progression as well as promote the development of metastatic disease. De Jaeger et al. [24] have observed the significant increase in early pulmonary metastases formation in mice with hypoxic primary tumors in KHT-C fibrosarcoma. It was concluded that hypoxic tumors are more likely to be able to metastasize and hypoxic environment might be implicated in metastatic ability of malignant tumors, including human ones, in particular, in soft tissue sarcoma [25, 26] and cervical carcinoma [27]. In particular, Nordsmark et al. [26] reported that hypoxia was an indicator for poor prognosis in soft tissue sarcoma patients but did not predispose to metastases independently of tumor grade. In this context, the important data were obtained demonstrating that the incidence of spontaneous pulmonary metastases in D-12 human melanoma xenografts was associated with the density of hypoxic foci in the primary tumor [21]. Authors suggested that there is an elevated probability of metastatic disease in patients developing primary tumors characterized by high density of hypoxic foci. It was shown recently that hypoxia may increase tumor cell metastatic efficiency [28].

Our data concerning positive correlation between activity of gelatinases in primary tumor and hypoxia level distinctly indicate that hypoxic environment in tumor tissue may induce enzymes which stimulate the metastasis. In this connection the data of Cuvier et al. [29] should be mentioned showing the increase of cathepsin content for KHT-LP1 sarcoma cells, exposed to hypoxia, and distinct correlation of such increase with the enhancement of the invasion ability of cells through Matrigel. At the same time it is necessary to intensify the studies, especially these on rodent tumors *in vivo* and on human tumors to detect the distinct and causal correlations between hypoxia level in primary tumor and both its MMPs activity and metastatic potential.

Overall, the present study clearly demonstrated that the increase of primary tumor volume is accompanied by the rise both of hypoxic fraction and activity of matrix metalloproteinases in primary tumor. It was also shown that enhancement both of hypoxia level and MMPs activity in primary tumor correlates with the rate of lung metastasis.

Moreover, it should be noted the curious regularity of some intratumoral events that was revealed in our study. It was detected that the activity of gelatinases as well as the number of lung metastases were drastically increased since the 18th day after tumor implantation (see Table 1). In particular, on the 18th day after tumor implantation the number of lung metastases was increased by a factor of 2.0, on the 20th day — by factor of 4.3, and on the 21st day — by a factor of 6.8 in comparison with that on the 16th day. These changes in the activity of gelatinases and metastasis were accompanied by the essential increase of hypoxia extent in primary tumor: on the 20th day Pi/βNTP ratio was increased by a factor of 1.75, on the 21st day — by factor of 2.0, and on the 24th day — by a factor of 3.7 in comparison with that on the 16th day. These data allow to suppose that the term between 18th and 24th days of primary tumor growth is the crucial time-point for the initiation of full metastatic cascade, including the increase both of hypoxia level and activity of gelatinases, as well as appearance of metastatic foci in the lung. It may be suggested that during 18th–24th days after tumor implantation the hypoxia level in primary tumor rises drastically and is accompanied by the induction of hypoxia-associated proteins, in particular matrix proteinases, resulting in the stimulation of rapid metastasis. Such suggestion should be proved in more extended studies to elucidate the mechanisms of activation of metastatic process and its causal association with intratumoral hypoxia.

REFERENCES

1. **Höckel M, Vaupel P.** Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 2001; **93**: 266–76.

2. **Brown JM, Ciaccia AJ.** The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. Cancer Res 1998; **58**: 1408–16.

3. **Pugh ChW, Ratcliff PJ.** Regulation of angiogenesis by hypoxia: role of the HIF system. Nature Med 2003; **9**: 677–84.

4. **Dachs GU, Tozer GM.** Hypoxia modulated gene expression: angiogenesis, metastasis and therapeutic exploitation. Eur J Cancer 2000; **36**: 1649–60.

5. **Buchler P, Reber HA, Lavey RS, Tomlinson J, Buchler MW, Friess H, Hines OJ.** Tumor hypoxia correlates with metastatic tumor growth of pancreatic cancer in an orthotopic murine model. J Surg Res 2004; **120**: 295–303.

6. **Chambers AF, Matrisian LM.** Changing views of role of matrix metalloproteinases in metastasis. J Natl Cancer Inst 1997; **89**: 1260–70.

7. **Engers R, Gabbert HE.** Mechanisms of tumor metastasis: cell biological aspects and clinical implications. J Res Clin Oncol 2000; **126**: 682–92.

8. **Vihinen P, Kahari V-M.** Matrix metalloproteinases in cancer: prognostic markers and therapeutic tagets. Int J Cancer 2002; **99**: 157–66.

9. **Chen PS, Zhai WR, Zhou XM, Zhang JS, Zhang YE, Ling YQ, Gu YH.** Effects of hypoxia, hyperoxia on the regulation of expression and activity of matrix metalloproteinase-2 in hepatic stellate cells. World J Gastroenterol 2001; **7**: 647–51.

10. **Cha HS, Ahn KS, Jeon CH, Kim J, Song YW, Koh EM.** Influence of hypoxia on the expression of matrix metalloproteinase-1, -3 and tissue inhibitor of metalloproteinase-1 in rheumatoid synovial fibroblasts. Clin Exp Rheumatol 2003; **21**: 593–8.

11. **Canning MT, Postovit LM, Clarke SH, Graham CH.** Oxygen-mediated regulation of gelatinase and tissue inhibitor of metalloproteinases-1 expression by invasive cells. Exp Cell Res 2001; **267**: 88–94.

12. **Himelstein BP, Koch CJ.** Studies of type IV collagenase regulation by hypoxia. Cancer Lett 1998; **124**: 127–33.

13. **Guo W, Ran Y, Wang G, Liu J, Yu L, Sun L, Yang Z.** Expression and hypoxic regulation of vascular endothelial growth factor and matrix metalloproteinase-9 in esophageal carcinoma. Zhonghua Zhong Liu Za Zhi 2002; **24**: 44–7.

14. **Bubnovskaya LN, Michailenko V, Kondrichin I, Osinsky S, Kovelskaya A, Levitin I, Sigan A.** Detection of tumor response to Co(III) and Fe(III) complexes by 31P-nuclear magnetic resonance spectroscopy. Exp Oncol 2002; **24**: 128–34.

15. **Maxwell RJ.** Application of nuclear magnetic resonance for investigation of the tumor microenvironment. In: Blood perfusion and microenvironment of human tumors. Molls M, Vaupel P, eds. Berlin, Heidelberg, New York: Springer-Verlag 1998; 145–60.

16. **Scott KA, Holdsworth H, Balkwill FR, Dlas S.** Exploiting changes in the tumor microenvironment with sequential cytokine and matrix metalloproteinase inhibitor treatment in a murine breast cancer model. Br J Cancer 2000; **83**: 1538–43.

17. **Moore S, Stein WH.** A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J Biol Chem 1954; **211**: 907–13.

18. **Okunieff P, Urano M, Kallinowski F, Vaupel P, Neuringer J.** Tumors growing in irradiated tissue: oxygenation, metabolic state, and pH. Int J Radiat Oncol Biol Phys 1991; **21**: 667–73.

19. **Fu K, Wendland MF, Iyer SB, Lam KN, Engeseth H, Lames TL.** Correlations between *in vivo* ^{31}P NMR spectroscopy measurements, tumor size, hypoxic fraction and cell survival after radiotherapy. Int J Radiat Oncol Biol Phys 1990; **181**: 1341–50.

20. **Vaupel P, Okunieff P, Kallinowski F, Neuringer LJ.** Correlations between ^{31}P-NMR spectroscopy and tissue O_2 tension measurements in a murine fibrosarcoma. Radiat Res 1989; **120**: 477–93.

21. **Rofstad EK, Halsar EF.** Hypoxia-associated spontaneous pulmonary metastasis in human melanoma xenografts: involvement of microvascular hot spots induced in hypoxic foci by interleukin 8. Br J Cancer 2002; **86**: 301–8.

22. **Airley R, Loncaster J, Davidson S, Bromley M, Roberts S, Patterson A, Hunter R, Stratford I, West C.** Glucose transporter Glut-1 expression correlates with tumor hypoxia and predicts metastasis-free survival in advanced carcinoma of the cervix. Clin Cancer Res 2001; **7**: 928–34.

23. **Loncaster JA, Harris AL, Davidson S, Logue JP, Hunter RD, Wycoff CC, Pastorek J, Ratcliffe PJ, Stratford I, West C.** Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 2001; **61**: 6394–9.

24. **De Jaeger K, Kavanagh M-C, Hill RP.** Relationship of hypoxia to metastatic ability in rodent tumours. Br J Cancer 2001; **84**: 1280–5.

25. **Brizel DM, Scully PS, Harrelson JM, Layfield LJ, Bean JM, Prosnitz LR, Dewhirst MW.** Tumor oxygenation predicts for the likelihood of distant metastases in human soft tissue sarcoma. Cancer Res 1996; **56**: 941–3.

26. **Nordsmark M, Alsner J, Keller J, Nielsen OS, Jensen OM, Horsman MR, Overgaard J.** Hypoxia in human soft tissue sarcoma: adverse impact on survival and no association with p 53 mutations. Br J Cancer 2001; **84**: 1070–5.

27. **Höckel M, Schlenger K, Arai B, Mitze M, Schäffer U, Vaupel P.** Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. Cancer Res 1996; **56**: 4509–15.

28. **Zhang L, Hill RP.** Hypoxia enhances metastatic efficiency by up-regulation Mdm2 in KHT cells and increasing resistance to apoptosis. Cancer Res 2004; **64**: 4180–9.

29. **Cuvier C, Jang A, Hill RP.** Exposure to hypoxia, glucose starvation and acidosis: effect on invasive capacity of murine tumor cells and correlation with cathepsin (L + B) secretion. Clin Exp Metastasis 1997; **15**: 19–25.

УРОВЕНЬ ГИПОКСИИ И АКТИВНОСТЬ МАТРИКСНЫХ МЕТАЛЛОПРОТЕИНАЗ-2 И -9 В КАРЦИНОМЕ ЛЕГКИХ ЛЬЮИС: КОРРЕЛЯЦИЯ С МЕТАСТАЗИРОВАНИЕМ

Цель: **изучить зависимость между гипоксией опухоли *in vivo*, активностью матриксных металлопротеиназ и метастатическим потенциалом опухоли.** ***Материалы и методы:*** **карцинома легких Льюис (3LL), перевиваемая на мышах C57Bl6. Общая активность матриксных металлопротеиназ (MMP-2 и -9) в опухоли измерялась биохимически, уровень опухолевой гипоксии оценивался с помощью 31P ЯМР-спектроскопии в перхлорных экстрактах ткани.** ***Результаты:*** **было показано, что уровень гипоксии в первичной опухоли резко повышается по мере роста опухоли и коррелирует с количеством метастазов в лёгких. Отмечена положительная корреляция между уровнем гипоксии и активностью MMP-2 и -9 в первичной опухоли. Установлено также, что активность MMP-2 и -9 в первичной опухоли прямо коррелирует с количеством метастазов в лёгких.** ***Выводы:*** **данное исследование показало, что рост первичной опухоли непосредственно сопряжен с увеличением уровня гипоксии в опухоли, который положительно коррелирует с активностью MMP-2 и -9 в первичной опухоли и с метастазированием.**